Characterization of Aluminium Stress Tolerance in Mung Bean for Crop Improvement

Summary of work

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SUMMARY OF WORK

(5000 words synopsis)

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1.0 Introduction:

Despite superior advancement in the fields of science and technology, farmers still have to fight different types of biotic and abiotic stresses to protect their crops. In addition to the biological processes along with half knowledge about the use of fertilisers, soil acidity is increasing day by day becoming a major concern for farmers. Aluminum toxicity- the main symptom of acid soil directly target root tip of plants causing loss of crop yields (Delhaize et al., 1995; Kochian et al., 2005; Ma et al., 2002). By inhibiting DNA synthesis (Morimura et al., 1978, Roy et al., 1989), by damaging the cytoskelon structure (Blancaflor & Gilroy., 1998; Alessa & Oliveira., 2001), by generating ROS (Yamamoto et al., 2002) and by decreasing the photosynthetic activity of plants (Bhalerao et al., 2013), Aluminum toxicity causes a greater loss in the field of agriculture. Plants have their own unique ways of dealing with the stress caused by Aluminum. Plants release organic acids like malate, citrate, oxalate on sensing Aluminum. These organic acids near the rhizosphere regions bind with and chelate Aluminum and prevent them from entering the plants (Ma et al., 1997 & 1998). However this supply of organic acids has to be continuous. The efflux of these organic acids were controlled by few genes namely ALMT1 (Aluminum Activated Malate Transporter1) for Malate and MATE (Multidrug And Toxic Compound Extrusions) for citrate. In case when Aluminum enters plants, they are taken up by ALS3 gene (Aluminum Sensitive 3) away from the sensitive tissues and sequester them in vacuoles making them inactive (Larsen et al., 2005). However all of these genes are regulated by STOP1 (Sensitive To Proton Rhizotoxicity1) gene (Sawaki et al., 2009). When STOP1 gene is mutated, the activity of ALMT, MATE, ALS3 genes were reported to be off, making the plants more sensitive to soil acidity and Aluminum stress in compared to the wild type plants.

ALMT1 genes however don't have any role in dealing with acid soil stress. Its function is only limited to Aluminum toxicity stress. However STOP1 gene control both of the stress factors by regulating other genes.

Besides the use of lime and traditional breeding methods which are expensive and limited in so many ways, use of transgenic approaches by introducing gene of desired interest in plants can definitely help to deal with Aluminum stress in a much better way and that is the purpose of this research.

Vigna radiata of legume family is very much rich in protein and essential nutrients and minerals and also easily affordable by common people, hence also known as poor men's meat (Hall et al., 2017, Singh et al., 2017). Not only this, mung bean has many medicinal properties like it is anticancerous, antihypertensive, hepatoprotective, antimelanogenesis, and immunomodulatory (Liyanage et al., 2018; Ali et al., 2014; Gupta et al., 2018; Chai et al., 2019; Xie et al., 2019; Lopes et al., 2018). No Aluminum stress tolerance studies performed in this economically important crop species. Hence, we planned to study the aluminium tolerance responses and transform this useful plant for better growth in Aluminum stressed conditions.

2.0 Objectives of study:

a) Cloning of aluminum stress responsive genes from *Vigna radiata*

b) Characterization of *Vigna radiata* seedlings against low pH and high Aluminum toxicities.

c) Development of Al stress responsive gene specific RNAi-transgenic plants.

d) Characterization of Al stress responsive RNAi-transgenic lines (T1) against different low pH and high Al^{3+} stress conditions and analysis of Al^{3+} tolerance mechanisms.

e) Development of different transgenic *Vigna radiata* by using the hyperactive AI^{3+} stress tolerant gene (anyone gene from *ALMT1, STOP1, ALS3, STAR1* and *MATE*).

3.0 Methodology:

3.1 Plant materials:

Mung bean seeds (*Vigna radiata* L. Wilczek cv. IPM-02-03) were used for this study. All the experiments were done in optimized plant tissue culture facility and the growth room was maintained by following the growth conditions; 14h (24°C) light and 10h (22°C) dark conditions. Light intensity of 200 μmol photons m^{-2} s^{-1} and 70% relative humidity was also maintained in the growth room.

3.2 Isolation of cDNA:

After the RNA isolation was done using RNA isolation kit, Nucleospin from Takara using oligo (dT) primers, cDNA synthesis was done with PrimeScript™ 1st strand cDNA synthesis kit (Takara). After that full length VrALMT cDNA was obtained by performing degenerate PCR along with RACE (Rapid Amplification Of cDNA Ends) methods (Sawaki et al., 2014; for degenerate primer). Then designing for the gene specific primers for 3'-RACE and 5'-RACE were carried out by following the sequence of degenerate PCR products. The cDNA fragments which were amplified were cloned in pMD20-T clone vector from Takara and then sequenced.

By following *AtALMT1 (At1g08430), AtSTOP1 (At1g34370)* , *AtMATE1 (At1g51340), AtALS3 (At2g37330)* and *AtSTAR1 (At1g67940)* cDNA sequences, all the orthologous genes were identified by performing searches in TAIR and GenBank (www.ncbi.nlm.nih.gov/genbank/). Then sequence analysis, amino acid alignment, and phylogenetic tree construction were performed using CLC sequence viewer software version 7.0.2

3.3 RNAi Vector Construction:

The *VrALMT-RNAi* cassette was cloned into pBI121 (Toyobo) vector by replacing the *GUS* gene. Firstly, the sense fragment was amplified by PCR methods and the sense (3’end) and antisense (5’end) fragments were cloned from cDNA of *VrALMT1* gene and the length of the above fragment was 310 bp; 952 to 1261) joined to first intron of Isocitrate dehydrogenase (*AtICDH*; *At1g65930*) sequence (Nakagawa et al. 2007). The *VrALMT-RNAi* cassette was driven by cauliflower mosaic virus (CaMV) 35S promoter, while NPTII gene was under the control of NOS promoter. After the confirmation of sequence orientation, the pBI121-*VrALMT*-RNAi vector was introduced into *A. tumefaciens* (EHA105) by the freeze–thaw method. Further, we followed shoot tip mediated mung bean transformation.

3.4 Mung bean transformation:

For mung bean transformation, we used the shoot apex from mung bean. The seeds were first surface sterilised in 0.1% $HgCl_2$ for 7 minutes and then rinsed in sterilised distilled water for 30 seconds. After that the seeds were soaked and stirred in 70% ethanol for 1 minute and then washed in sterilised distilled water for 7 times, one minute each to get rid of traces of chemicals used. Now the sterilised seeds were inoculated in media for germination [MS+BAP (1.0 mg/L)] and were kept in dark for 2-3 days in 25°C. After the seeds germinated the plants were kept in light (16h) and dark (8h) under the same temperature for 3 more days. Then after the plants were formed completely, under sterilised condition the shoot tips were excised for transformation studies. In the mean time, the transformed *A. tumefaciens* cells were harvested and then resuspended in 10 mL liquid infection media made of calcium-free MS basal medium (pH 5.2) supplemented with BAP (1.0 mg/L), sucrose (10 g/L), acetosyringone (100 mg/L) and pluronic F68 (0.02%). The excised shoot tips were then placed in bacterial suspension for 20 minutes and agitated minimally. Then after quick drying in sterilised filter papers, the explants were co-cultivated on a co-culture medium [MS + BAP (1.0 mg/L), 2iP (1.0 mg/L), sucrose (30g/L), acetosyringone (100mg/L), gelrite (2.0 g/L)] and placed at 28°C under dark conditions for 4 days. Then the newly developed shoots were washed with sterilised water containing carbenicillin (500 mg/L) and placed in to a new media (2 mg/L BA, 30 g/L glucose, 2 g/L gelrite, 250 mg/L carbenicillin, 100 mg/L Kanamycin) for pre-selection. The cultures were kept in 16h per day light conditions and temperature of 25°C and sub cultured every week for 28 days.

After a period of 28 days, the healthy shoots which were approximately 2 cm in height were excised aseptically from the newly developed multiple shoot cultures and inoculated to root induction media (MS basal + 0.75 mg/L IBA). Followed by 28 days of culture, the plants with well developed roots measuring minimum 4 cm in length were washed properly to get rid of media and then hardened and maintained for 3 weeks with proper care in culture room. After hardening procedure, the plantlets were transferred to plastic pots containing 1:1 mixture of sand and vermiculite (and maintained in high humid conditions).

3.5 Overexpression of Vector Construction and mung bean Transformation:

The *AtALMT* overexpression vector was designed by replacing the *GUS* gene of the binary vector pBI121 (Toyobo) with the *AtALMT1* sequence. The binary vector pBI121 which was carrying Arabidopsis *AtALMT1* was under the control of CaMV35S promoter while NPTII gene was under the control of NOS promoter. After the confirmation of gene orientation by sequence analysis, the

vector was introduced into *Agrobacterium tumefaciens* strain EHA105 and transformed into WT mung bean plants by the using shoot apex explants (Clough and Bent, 1998).

3.6 Molecular analysis of transgenic plants and seed collection:

PCR positive transgenic plants were used further for molecular confirmation. Genomic DNA isolation was performed from young leaves of transformed (T0 generation) and wild-type (WT) mung bean plants by using PureLink® PlantTotal DNA Purification kit from Invitrogen. For all transgenic plant lines, T1 seeds were collected from PCR positive transgenic plants (T0) were planted on half-strength Murashige and Skoog medium and 50 mg/L Kanamycin. The PCR mixture was prepared and performed with Prime STAR Max DNA Polymerase (R045B, Takara, Japan). PCR amplifications were performed at the following conditions: 92˚C for 4 min; followed by 30 cycles of 92˚C for 1 min; 10 cycles of 60°C (-0.5˚C step-down) for 15 sec, 26 cycles of 55˚C 15 sec, 72˚C for 30 sec; and a last elongation step of 72˚C for 3 min. The amplicon obtained had 320 bp. The PCR positive transgenic plants were then placed to plastic pots containing mixture of soil and vermiculite with the ratio of 1:1 (w/w) in room temperature (25°C) under 16 h of day light to collect T1 seeds. The T1 transgenic seeds were collected and transgenic seedlings were selected on MS (Murashige and Skoog, 1962) selection media containing MS salts, B5 Vitamin, 30 g/L glucose, 100 mg/L Kanamycin, 8.0 g/L agar at pH 5.8. 3 days old, Kanamycin tolerant transgenic seedlings were considered for further experiment. To perform southern blot analysis, genomic DNA was isolated from the leaves of plants grown in green house, digested with HindIII. DNA (20 µg/lane) was separated by using 0.8 % agarose gel electrophoresis and transferred to Amersham Hybond XL membranes (GE Healthcare). Southern hybridization was performed using a non-radioactive DNA labelling and detection protocol.

3.7 Hydroponic Culture and Stress Treatments:

The WT and transgenic mung bean seeds were grown aseptically in seed beds. The germinated seeds of each line were placed on a polystyrene float on culture solution. The basal solution contained 2% (w/v) MGRL nutrients and 200 mM $CaCl_2$ without inorganic phosphorus (Kobayashi et al. 2007). For studying Al and low pH stress, Al^{3+} and H^+ toxic solutions were made by adjustment of pH with HCl and then adding $AlCl_3$ to obtain concentrations (from 10 µM to 50 µM) known to be toxic to mung bean seedlings (pH 4.4 for H^+ and pH 4.75+20 µM for Al^{3+} toxicity). Hydroponic solution at pH 5.5 without Al was used as the control to evaluate Al^{3+} and H^+ toxicity. Further, mung bean seeds were surfaced sterilised with 70 % ethanol for 4 min, were stratified at 4°C for 3 days and planted on tissue culture containers (PW1301, Himedia, India). Further, the

seedlings were transferred to 1/5 Hoagland nutrient solution along with 1 mM MES and 0.8% agar (pH 5.5). After 7 days, the grown healthy seedlings of uniform size were selected for Aluminum stress studies. All solutions where the seedlings were placed were renewed every 2 days for 14 days at 25°C. After collecting the T1 seeds, five to seven independent RNAi lines / Overexpression lines were selected for each treatment.

3.8 Expression Analysis:

Real-time RT-PCR for quantitative analysis was performed with Power SYBR Green (Applied Biosystems) and an ABI PRISM 7000 instrument (Applied Biosystems) based on the manufacturer's protocol for the standard curve method. For the experiments, a diluted version of cDNA series was used to get standard curves of target genes and the internal control.

3.9 Morphological Characterization of Transgenic Plants:

To study root architecture, mung bean roots were cleaned and placed on graph sheets and analyzed (Obiefuna and Ndubizu 1979). By using ImageJ software, the top 7 lateral roots were used to calculate the mean value of lateral root. To analyse the secondary root growth variations, 7d or 14 d old seedlings were used and treated with low pH and high aluminum stress conditions. After growing for 7 more days, the length of primary root was then calculated to the nearest millimeter with the help of a ruler. The number of lateral roots on each primary root was determined by counting all lateral roots that had grown from the primary root (Rashotte et al., 2001). The variation in root growth pattern was also recorded by using ImageJ software.

3.10 Collection of Root Exudates, and Quantification of Malate:

Mung bean seedlings were grown aseptically in hydroponics solution containing 100 mL of 1/50 MGRL nutrient with 200 mM $CaCl_2$ solution along with 1% Sucrose (pH 5.5) in plastic pots for 7 days. To study stress treatment, selected lines were given Aluminum stress with 20µM $AlCl_3$ (pH 4.75) or low pH stress (4.4). Malate was quantified by the enzyme reaction method (Hampp et al., 1984).

3.11 Hematoxylin Staining:

Mung bean seedlings were grown for 1 week in 100 mL of 1/50 MGRL nutrient with 200 mM $CaCl_2$ solution along with 1% Sucrose under aseptic conditions (pH 5.5) in plastic pots. To study the Al accumulation, selected lines were treated with 20µM $AlCl_3$ (pH 4.75) stress for 5 mins,

then put in 0.2 % iron hematoxylin and 0.02 KIO_3 for 5 mins (Polle et al., 1978). The excess dye was removed by washing the roots in deionized water under aeration for 15 minutes.

3.12 Statistical Analyses:

For phenotype scoring, uniformly germinated mung bean seeds were collected from the seed germination bed and transferred to hydroponics conditions. For the stress treatment and analysis reasonable numbers (n=5-15) of healthy seedlings were selected. For all control plants ~15 seedlings were considered for phenotypic analysis. For all the transgenic plants minimum 5 (n=5-7) seedlings were considered for the analysis. The experiments were repeated 5 times (for Wild type or non-transgenic plants). We recorded the plant phenotypes according to the code number belonging to each treatment group. Three important Al stress responsive agronomic quantitative traits root length, secondary root length and dry weight were also recorded. Further, the data were transferred to SPSS software to calculate mean and standard error values. Means and standard errors were performed throughout the study and the values were calculated and analysed (n = 5-15) (Snedecor and Cochran, 1989). The data were also analysed for variance by using Duncan's Multiple Range Test (DMRT) with the help of the SPSS statistical programme version 7.0 in necessary places.

4.0 Results and Discussions:

Acid soil is considered to be phytotoxic because of the unavailability of essential nutrients for plants like Calcium, Phosphorus, Molybdenum and Magnesium and also because of increased solubility and toxicity of Al^{3+} and manganese ions (Ritchie, 1998; Hede et al., 2001; Taye, 2007). Every time pH drops from 5.5 to 4.5 and from 4.5 to 3.5, amount of solubilized Aluminum increases 1000 times more, proving to be more lethal for the plants. Hence, some plants like wheat that can manage the stress at pH 5 gets completely destroyed when the pH drops to 4. That's why even a slight change in pH can completely destroy a plant. Hence, soil acidity and aluminum toxicity is becoming a worldwide social and economic concern.

In the last few years, there is serious improvement in the fields of plants molecular biology. Studies were done on the functional characterization of gene that are linked to a particular trait or traits (Baulcombe, 2004; Meister and Tuschl, 2004; Pathak and Gogoi, 2016). By taking advantages of these improvements, we decided to characterize ALMT1 gene in mung bean.

OBJECTIVE 1

4.1 Molecular cloning and characterization of aluminum stress responsive genes (*ALMT1, MATE, STAR1, STOP1* or *ALS3*) from *Vigna radiata* by Degenerate, RACE-PCR methods

In Arabidopsis plants, ALMT and MATE genes are mainly involved organic acid release and regulates Aluminum resistant/tolerant. ALMT1 genes are regulating malate extrusion and MATE1 genes regulating citrate extrusion under acid soil stress conditions. The STOP1 system is regulating the ALMT1 and MATE genes and the STAR1 genes are controlling the functions of STOP1 by binding with the promoter region. Moreover, the ALS3 genes are possibly involved in Al transport in the leaf tissue (Tiwari et al., 2014; Ryan et al., 2009; Ligaba et al., 2006).

Based on these studies, we planned to identify the key gene which will regulate the Al tolerance mechanism of mung bean plants. To clone the different Al stress responsive genes (ALMT1, MATE, STAR1, STOP1 or ALS3), firstly, we designed different degenerate primers and amplified the conserved domain of the above genes. By using the conserved domain, gene specific primers were designed for RACE-PCR studies. Based on the RACE-PCR results, full length sequence of the ALMT1, MATE, STAR1, STOP1 and ALS3 genes were identified (Gel images enclosed in the presentation). To identify the key gene involved in Al tolerance of mung bean plants, we developed hydroponic cultures with or without Al ions under acid soil stress conditions. The results indicated that ALMT1 expression is significantly enhanced under Al ions stress conditions when compared with other Al stress responsive genes. STOP1, STAR1 and AL3 expression profiles also enhanced under acid soil stress conditions. Further, we also noticed that the MATE expression was activated only under the Al ion stress conditions. Interestingly, STAR1 expression was detectable under control and Al stress conditions but not with that much variation. Further, we studied the citrate and malate contents exudated by the mung bean plants under Al stress conditions. The results indicated that these plants are producing malate to tolerate the Al ions present in the hydroponic media **(Fig.1 a&b)**.

Based on these studied, we aimed to characterize the *VrALMT1* genes in mung bean plants by using transgenic approach.

OBJECTIVE 2

4.2 Aluminum tolerance mechanism of mung bean plants

There are reports in wheat (Sasaki et al. 2004), *Arabidopis thaliana* (Kobayashi et al., 2007), Rapeseed (*BnALMT1*) (Ligaba et al. 2006), Maize (*ZmALMT1*) (Pineros et al., 2002), Barley (*HvALMT1*) (Gruber et al., 2010), Sorghum (*SbMATE*) (Furukawa et al., 2007; Magalhaes et al., 2007) and Cotton (*GhMATE1*) (Kundu et al., 2019) plants on sensing aluminum stress release

organic acids like malate and citrate that chelate with Aluminum, making Al non-toxic and preventing it from entering plants. ALMT and MATE genes are mainly involved in releasing these organic acids and dealing with the stress but the Aluminum resistant/tolerant genes like ALMT1 are not yet characterised in mung bean. So we planned to characterise ALMT1 gene from mung bean plants to study the role of the gene and how much efficient it is in dealing with the Aluminum toxicity stress.

To study the aluminum tolerance of mung bean plants, 3 days old-seedlings were subjected to different pH (5.5 to 4.0) and Al (5 to 100 uM of $AlCl_3$) treatments (**Fig.2 & 3**). After 4 to 14 days of stress treatments, the variations in the shoot and root growth parameters were recorded. The results indicated that mung bean plants are very sensitive to moderate and high Al stress condition (20-30 µM at pH 4.75). Under high concentrations of Al treatments (40 to 50 uM), the seedlings showed no growth. Particularly, primary root length, secondary root length, total biomass were significantly decreased under high proton and/or Al stress conditions. based on these results, for further studies, we selected 20µM at pH 4.75 for the stress treatments of transgenic plants.

Proline content was also measured from control and stressed plants (Al stressed and low pH stress). Proline is known to be accumulated in plants as the stress factor increases (Rhodes et al., 2002; Naidu et al., 1991) and reported to protect plants against different stresses (Bassi et al., 1993; Costa et al., 1994; Alia et al., 1991; Talanova et al., 2000). In particular, they form phytochelatins that chelate heavy metals and making them non-toxic (De Knecht et al., 1994). In our experimental results, Proline accumulation increases as the pH lowers to 4.5 and 4.25 and similarly as the Al^{3+} stress increases when compared with the control.

OBJECTIVE 3

4.3 RNAi Vector construction

ALMT1 family genes contribute Al resistance of several plant species *via* organic anion efflux (malic acid). Moreover, the ALMT1 gene expression was activated only under Al^{3+} stress conditions (Liu et al. 2009). By following standard procedures, we amplified the full length of *VrALMT1*. The functional cDNA sequence of *VrALMT1* consists of 1437 bp which encodes 478 aa residues. The possible number of transmembrane helices in *VrALMT1* was also studied by using TMpred. It revealed that 7 inside to outside and 7 outside to inside helices are present in *VrALMT1* system. Multiple alignment of *VrALMT1*, displayed 76 to 99% similarity with other species of legume plant members (**Fig.4**).

The *VrALMT1-RNAi* vector was prepared by replacing the GUS gene of pBI121 (Toyobo) with the *VrALMT1-RNAi* cassette. For RNAi cassette preparation, the sense and antisense sequence

consisted of the partial cDNA of *AtALMT1* (310 bp) joined to first intron (435 bp) of the Arabidopsis *Isocitrate Dehydrogenase* (ICDH) (AT1G65930) sequence (Nakagawa *et al*. 2007) (**Fig.5**). The *VrALMT1-RNAi* cassette was under the control of Cauliflower Mosaic Virus (CaMV) 35S promoter, while *nptII* gene was under the control of NOS promoter. After the confirmation of gene orientation by sequence analysis, the *pBI121- VrALMT1-RNAi* vector and control vector (pBI121) were introduced into *A. tumefaciens* (EHA105) by the freeze–thaw method (Pan *et al*. 2017). Further, shoot tip culture mediated transformation procedure was adopted for the development of transgenic plants (**Fig.6**).

OBJECTIVE 4

4.4 Characterization of ALMT1-RNAi plants under acid soil stress conditions

Plant genome sequencing have provided us with lots of sequence information in plants. However functions of many genes are still not known including model plant Arabidopsis. So a powerful tool was needed that can be used to understand gene function. To attain this goal many genomics functional tools were developed in the last few years. RNAi (RNA-interference) is one such tool that helps to understand gene function. The gene of interest gets silenced by RNAi mediated pathway in order to study the role of the gene in plants. The prospect of RNAi is useful for both functional genomics and agricultural applications like improving crops overcoming stress challenges (Senthil-kumar., 2010). As we have mentioned no work has been done on mung bean regarding characterization of Aluminum resistant genes, so we planned to use RNA-i pathway to knockdown our gene of interest to understand the role of it associated with the stress.

We developed transgenic mungbean plants with *VrALMT1-RNAi* construct by *Agrobacterium tumefaciens* -mediated transformation by infecting the shoot apex explants. The kanamycin tolerance putative transgenic plants were further selected for PCR confirmation. The T1 seeds were collected from the PCR positive T0 transgenic plants were allowed for self-pollination. Furthermore, Kanamycin tolerant 3d old seedlings (KD lines) were selected for Al stress tolerance studies. For the Al stress tolerance studies, vector control plants (WT) were considered as control plants and compared with *VrALMT1-KD* (Al-untreated and treated [UT & T]), and *VrALMT1-OX* (Al-UT & T) plants.

Under Al stress conditions, transgenic plants showed repressed expression of *VrALMT1* and displayed very sensitive phenotypes. The stress assay was made for long time exposure under hydroponic conditions. All the seedlings were treated for 7 days and compared with the control plants. (**Fig.7**).

Under control conditions (pH 4.75), all KD lines showed no significant root growth variations when compared with control plants. Besides, under Al (pH 4.75 + 20µM) and proton (pH 4.4) stress conditions, we observed significant decline in the primary and secondary root length of RNAi transgenic lines. Al sensitivity was significantly enhanced in *VrALMT1* down-regulated lines. After 7 days' treatment, under Al stress conditions (pH 4.75 + 20µM), the transgenic lines exhibited only 21 % inhibition in the primary root length when compared with control plants. Moreover, the primary root growth was ~62% inhibited for RNAi plants under high Al toxicity (pH 4.75 + 25 µM) treatments. Besides, we also observed retarded secondary root initiation and growth process in KD plants under high Al rhizotoxicity conditions. For the secondary root initiation studies, all the seedlings (control and transgenics) were maintained under control conditions (pH 5.5) for 4 days. For Al stress treatments, the seedlings were transferred to different stress conditions. After 12 days' growth, we observed significantly reduced secondary root growth between RNAi transgenic and WT plants under Al toxic conditions (pH 4.75 + 20µM). In particular, Al stress displayed nearly 41% decline in the secondary root length of *VrALMT1* suppressed lines than in control plants. On the other hand, under proton stress conditions no significant root growth variations were recorded in RNAi seedlings. These results suggest that *VrALMT1* is necessary for Al tolerance mechanisms by regulating the primary and secondary root growth. Only limited number of reports showed that Al stress also inhibits secondary root growth (Kundu et al., 2019; Kundu and Ganesan, 2020). In barley, Al toxicity inhibited the growth of newly-emerged lateral roots (Hecht-Buchholz and Foy 1981; Tamas et al., 2003). Besides, the root hair numbers were significantly decreased in Al treated Clover plants (Care., 1995). Recently, Wang et al. (2017) studied Al^{3+}-induced IAA transport in alfalfa plants and their results indicated that the Al^{3+}-induced reduction of root growth could be associated with the inhibitions of IAA synthesis in apical buds and imbalanced distribution of IAA in root tips.

4.5 Repression of Al-Activated malate excretion

Under Al stress conditions (pH 4.75, 20 µM for 48h), the wild type plants secreted highest concentration of malate when compared with Al treated control plants. Moreover, all RNAi transgenic lines showed suppressed levels (36 %) of Al-inducible malate than in control plants. These results suggested that the repressed secretion of malate is one of the key Al tolerance mechanisms controlled by *VrALMT1*. Several reports proved that Al tolerance is achieved by organic acid release into the rhizhospehere (Kundu et al., 2019; Kundu and Ganesan, 2020). Miyasaka et al. (1991) showed that an Al-resistant cultivar *Phaseolus vulgaris* excreted eight-fold increase in citrate excretion than Al-sensitive cultivars. Moreover, overexpression of citrate synthase resulted in enhanced Al resistance (de la Fuente et al., 1997; Koyama et al., 2001). Moreover, we

measured the amount of Al accumulation by using hematoxylin staining. Both RNAi plants and control were treated with Al (20 µM pH 4.75) for 15 mins and the variations in the density of purple color were recorded. The RNAi plants displayed denser purple staining than in control plants which indicate greater Al accumulation. The above result stating that ALMT1 expression is essential to control the Al accumulation in the root system.

In continuation of these results, we studied the variations in the expression profile of different genes which are responsible for Al tolerance mechanisms. We evaluated the expression profile of STOP1, MATE1, and ALS3 in mung bean plants. The STOP1 (*Sensitivity to Proton rhizotoxicity 1*) is one of the essential transcription factors required for regulation of Al^{3+} and H^{+} tolerance mechanisms and regulating the expression of organic acid excretion (*AtALMT1* and *AtMATE*) (Iuchi et al. 2007). The transcript levels of all the above genes were not affected in Al-treated lines and both WT and RNAi lines displayed almost same expression profile under Al stress conditions and this result suggests that the expression of all the above genes are not regulated by ALMT1.

OBJECTIVE 5

4.6 Overexpression of *AtALMT1* in mung bean plants

Our results highlighted that the *AtALMT1*-overexpression displayed enhanced root growth, under control and Al stress conditions. We also recorded enhanced malate excretion in transgenic plants than in WT plants. The 3 days old Kanamycin tolerant T1 transgenic seedlings were subjected to Al stress (pH 4.75, 20.0 µM $AlCl_3$) studies. After 3 days of maintenance in hydroponic media, both WT and overexpressor plants were treated with Al (4 days). We noticed significant variations in the seedling development. In Particular, approximately 55% decline in the primary root length of WT plants were recorded under Al stress condition when compared with WT untreated plants. Besides, *AtALMT1* overexpressors displayed significantly enhanced the root growth under Al stress conditions when compared with Al treated WT plants. Moreover, the *AtALMT1* transgenic plants exhibited ~12% increase in the primary root growth under control conditions (without Al) (**Fig.**8).

In our earlier studies, we observed that *GhSTOP1* and *GhMATE1* are involved in Al tolerance levels and controls the primary and secondary root growth under Al stress conditions in cotton plants (Kundu et al. 2019; Kundu and Ganesan, 2020). Our present study pointed out that the ALMT family gene (*AtALMT1*) is also involved in Al tolerance mechanisms and regulating primary and secondary root growth.

5.0 Conclusion and future prospective:

Under acid soil stress conditions (pH 3- 5; Al concentration 10 µM to 100 µM), the crop plants are facing several growth defects. Particularly retarded root growth and low yield are the very common problems. Variations in the Al concentrations play an important role in plant species distribution under natural environment conditions. There are several genes and transcription factors are involved in Al detoxification mechanisms. In particular, the organic acid transporters have been implicated in detoxification mechanisms of Al and heavy metals, rhizosphere microbial diversity, disease resistance, nutrient homeostasis (Li et al., 2002; Liu et al., 2016; Wang et al., 2017). Although the use of lime in soil can detoxify the stress and prevent the solubilization of toxic Al ions but practically it will take a long time. Hence, the best way to deal with the present-day Aluminum toxicity stress problems is to use Al tolerant cultivars along with the application of limes.

Our studies narrated that ALMT1 expression is not Al dependent but are activated under Al stress conditions and necessary for Al tolerance mechanisms by regulating different genes which are responsible for root growth and citrate excretion. Our results also pointed out that the overexpression of Arabidopsis ALMT1 enhances the Al tolerance mechanisms by enhancing the malate excretion and root growth. Based on these observations, we suggest that *AtALMT1* could be useful to develop Al tolerance transgenic crops. Further, we also observed that the downregulation or overexpression of ALMT1 in mung bean displayed significant variations in the root nodule formation (unpublished). Further studies on root nodule formation under Al stress conditions may provide some valuable information for the crop improvement program.

6.0 Achievements:

S. Das* and M. Ganesan, (2022), Aluminum induced malate transporter (ALMT1) is regulating the Aluminum stress tolerance responses of mungbean seedlings, *Plant Gene*. 32. (Published article in Journal *Plant Gene*).

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Journal Publications:

1. A.Kundu, **S. Das***, S. Basu, Y. Kobayashi, Y. Kobayashi, K. Hiroyuki, M. Ganesan, (2019) *GhSTOP1*, A C_2H_2 type zinc finger transcription factor is essential for Aluminum and proton stress tolerance and lateral root initiation in cotton, *Plant Biol. J*. 21 , pp. 35-44. https://doi.org/10.1111/plb.12895 **Equal first author contribution*

2. **S. Das*** and M. Ganesan, (2022) Aluminum induced malate transporter (ALMT1) is regulating the Aluminum stress tolerance responses of mungbean seedlings, *Plant Gene*, 32, 100388. https://doi.org/10.1016/j.plgene.2022.100388

Presentation and participation at academic conferences/ meetings:

1. **S. Das**, A. Kundu, M. Ganesan, Cloning and characterization of aluminum stress responsive genes in mung bean plants. **Poster** presentation in International conference on Allied And Applied Biotechnology (ICAABT-2017) organized by Biologix Research and Innovation Centre Pvt. Ltd. (BRICPL), India during April 01-02, 2017 in Orchaa, MP (India). Received **BEST PRESENTATION AWARD** under poster category.

2. **S. Das**, M. Ganesan, Effect of aluminum toxicity in mung bean plants. **Poster** presentation in UGC-CPE sponsored International conference on Facets of Basic Sciences and Applications (FBSA) organized by IQAC and Departments of Botany, Chemistry, Computer science, Electronics, Mathematics, Micro-Biology, Physics, Statistics and Zoology, during February 5-7, 2018 at Bijoy Krishna Girls College, Howrah.

3. **S.Das**, Participated on Plenary session on Food Security and Genetically Modified Crops organized by West Bengal State Council of Science and Technology and Department of Biophysics, Molecular Biology and Bioinformatics, Calcutta University during 25th July, 2013, catalyzed and supported by National Council for Science, Technology and Communication, Department of Science and Technology, Govt. of India.

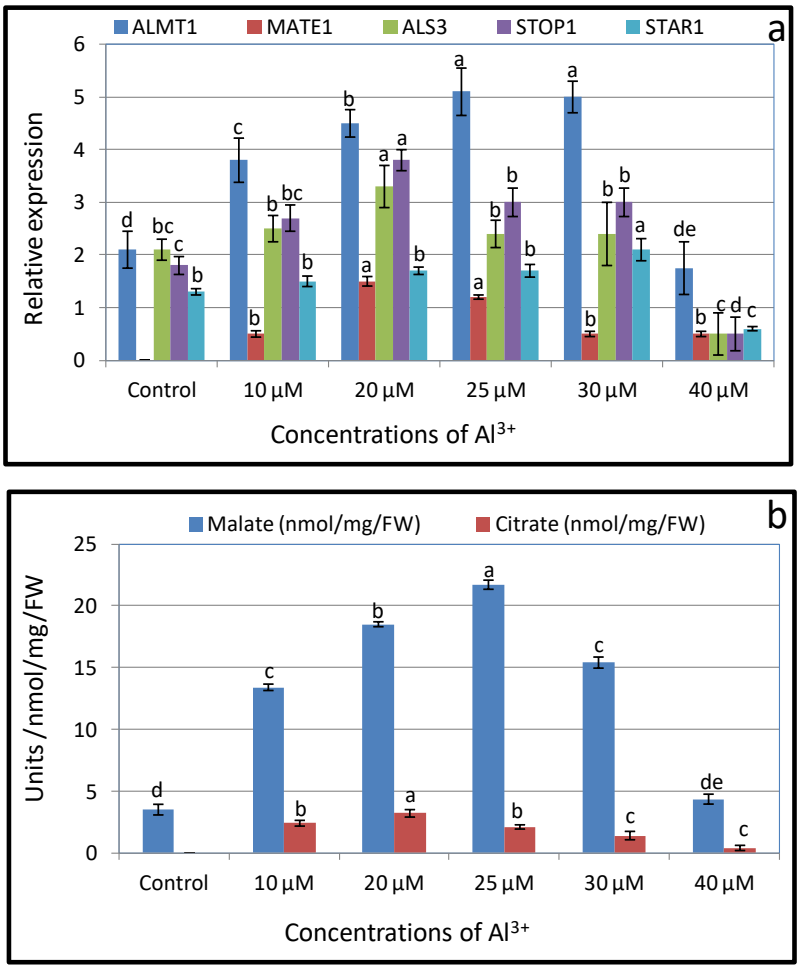


Fig.1

a) Variations in the expression profile of different Al stress responsive genes (STOP1, ALMT1, MATE1 And ALS3) under acid soil stress (pH4.75) conditions. Analysis was made by using the control and Al treated seedlings (10 days old) and actin was used as the internal control.

b) The impact of different concentrations of Al on organic acid excretion from the mung bean seedlings (10 days old).

Each value indicates the mean ± SE. Mean values within a treatment followed by the same alphabets are not significant at P = 0.05 according to DMRT.

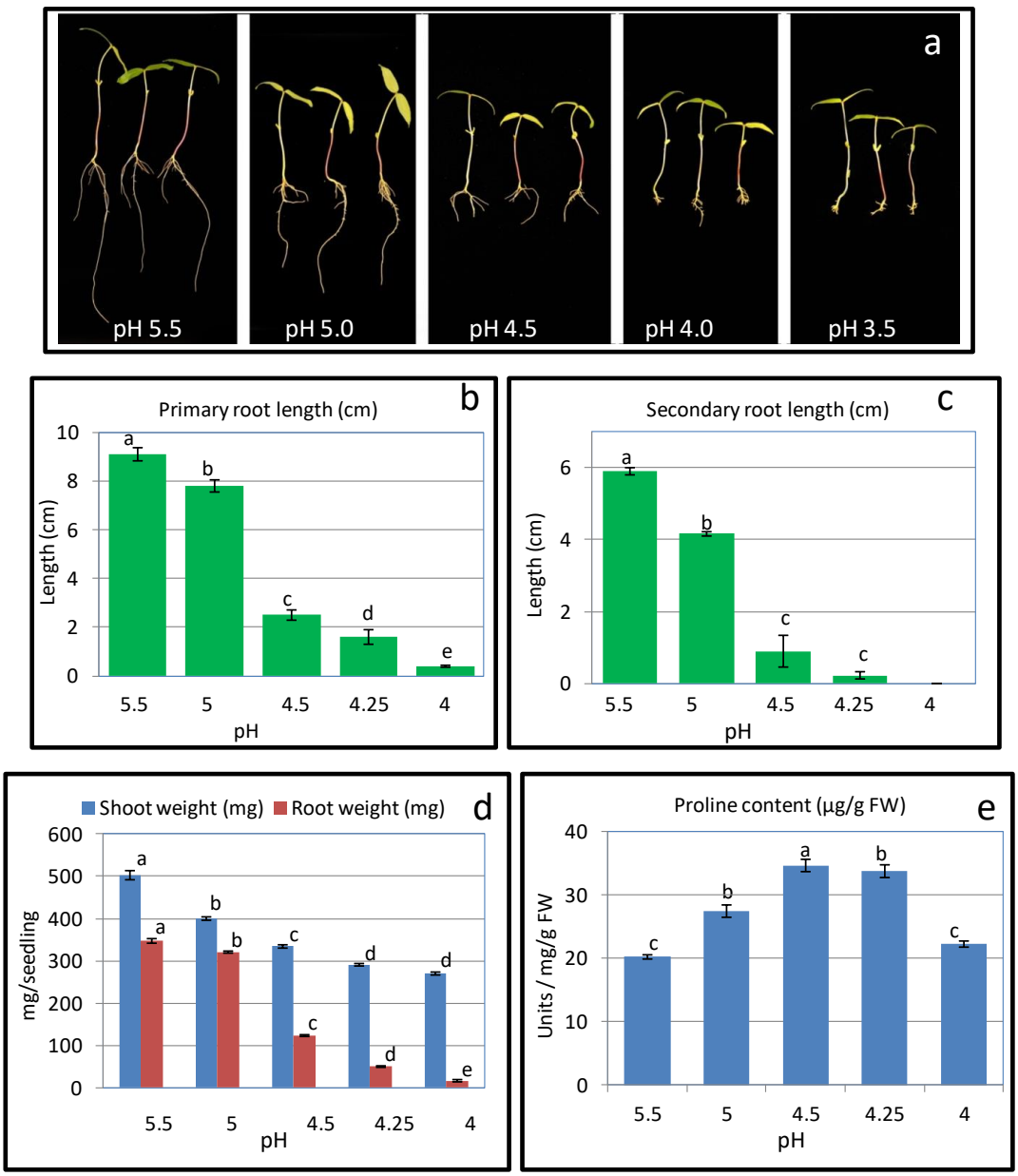
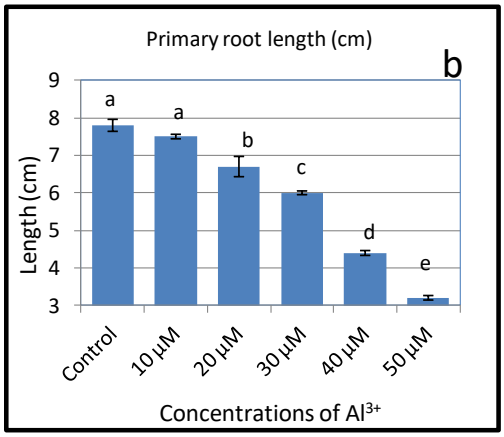
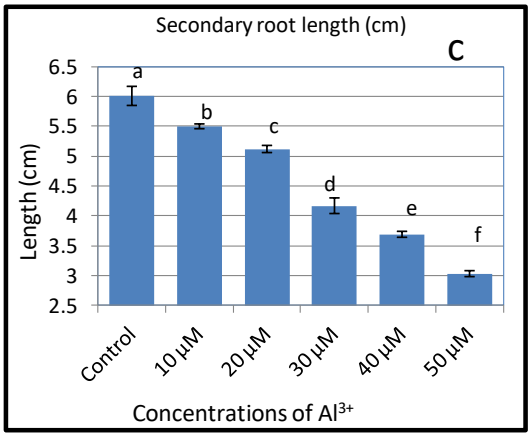
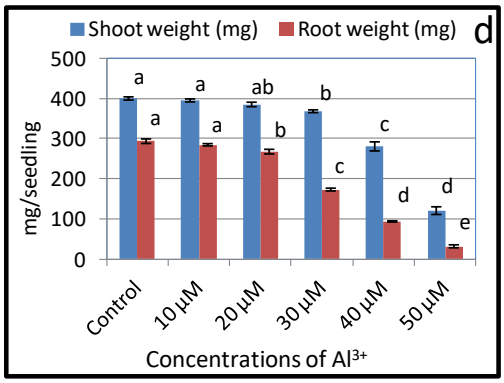


Fig.2

The effect of different pH (proton stress) on growth and development of mung bean seedlings under hydroponics (MGRL) conditions. Three days old germinated seeds were transferred to the ports containing hydroponic mediated and then the pH was adjusted by using NaOH or HCl according to the stress treatment. After the stress treatments, primary root length from 7 old seedlings (b), secondary root length (c), dry weight (d) and proline content (e) were measured from the 14 days old mung bean seedlings. Each value indicates the mean ± SE. Mean values within a treatment followed by the same alphabets are not significant at P = 0.05 according to DMRT.







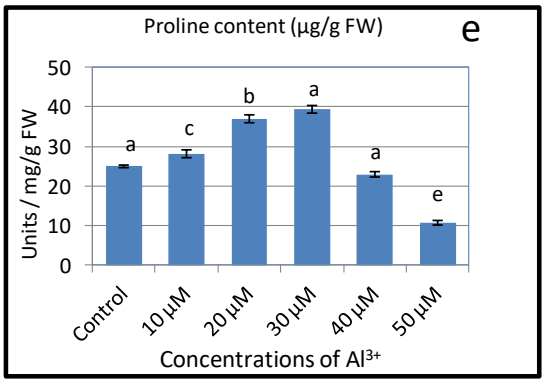


Fig.3

The effect of different concentrations of Al ($AlCl_3$) on growth and development of mung bean seedlings under hydroponics conditions. After 5 days treatment (a), primary root length (b) of seedlings were measured. After 10 days treatment, secondary root length (c), total biomass (d) and proline contents (e) were measured. Each value indicates the mean ± SE. Mean values within a treatment followed by the same alphabets are not significant at P = 0.05 according to DMRT.

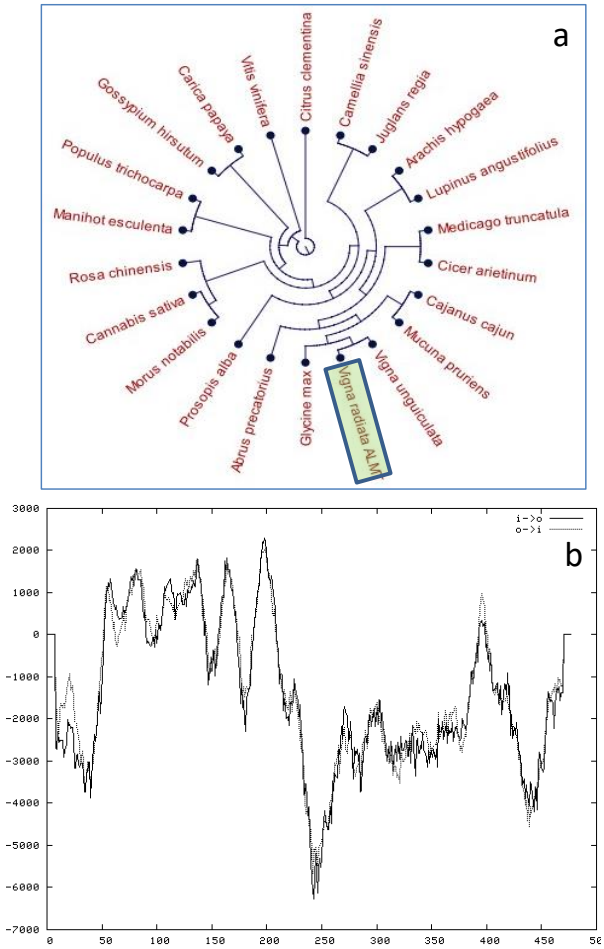


Fig.4

a) Phylogenetic tree analysis studies showed that VrALMT1 is closely related to the ALMT1 of legume family plants. Particularly, *Vigna unguiculata* showed amino acid sequence similarity with VrALMT1.

b) Possible transmembrane helices predicted by using TMpred. Totally seven inside to outside helices and seven outside to inside helices were recorded. The sequence positions in brackets denominate the core region. Only scores above 500 are considered significant.

a

ATGGCATCTCCAAACACAAACCAGGAGAAATCAGGGGTGCTTGGTCGAGTATTGGCCTTGCCAAAAGTTCTTAAGAAGAAGGTCTTGGGAATTTGTAGG
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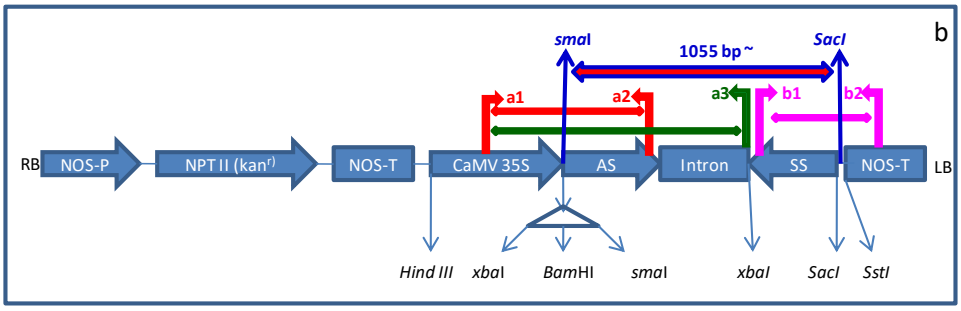

Fig.5

(a) The target of VrALMT1 sequence used for RNAi vector construction.

(b) Partial cDNA of VrALMT1 sandwiched with the first intron of AtICDH (isocitrate dehydrogenase), and the gene construct was driven by the cauliflower mosaic virus 35S promoter (CaMV35s) with kanamycin selection marker (NPT II). The primers used for the construction / confirmation of RNAi cassette were highlighted

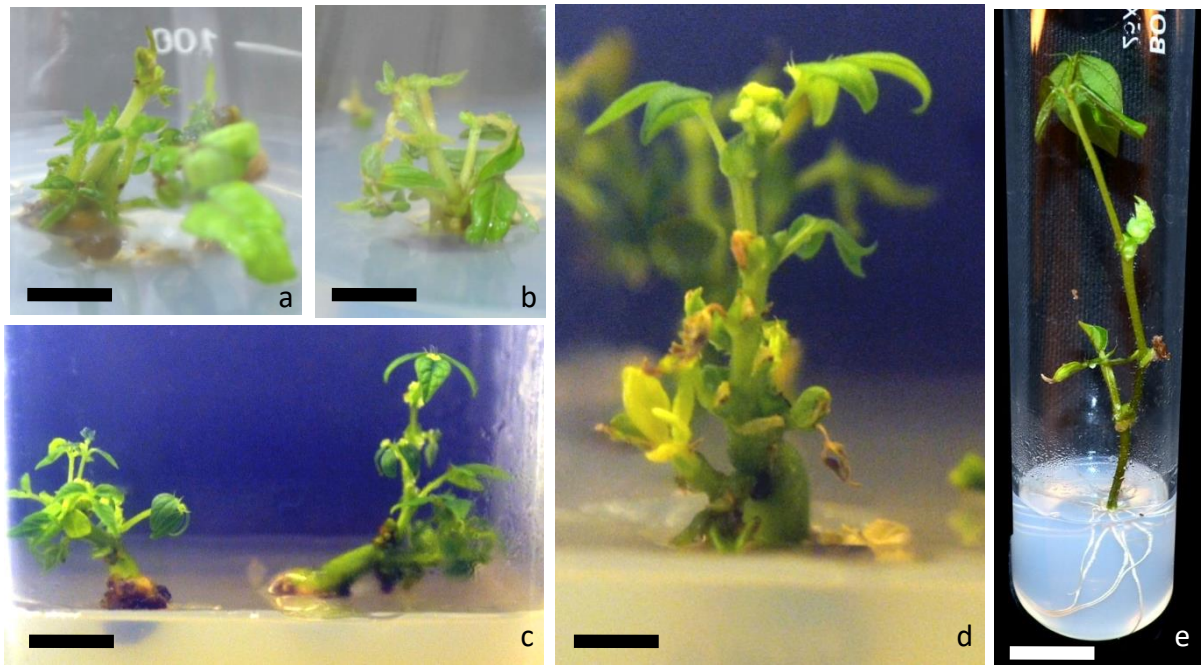

Fig. 6
In vitro regeneration of mung bean plants by using shoot tip explants. (a & b) 30 days old multiple shoots obtained from shoot tip explants. *Bar= 1 cm.* (c) 15 days old shoots in shoot elongation in GA_3 fortified media. *Bar= 1.5 cm.* (d) 30 days old shoot in shoot elongation media. *Bar= 1.25 cm* (e) Fully developed plantlet with well developed shoot and root system. *Bar= 1.25 cm*

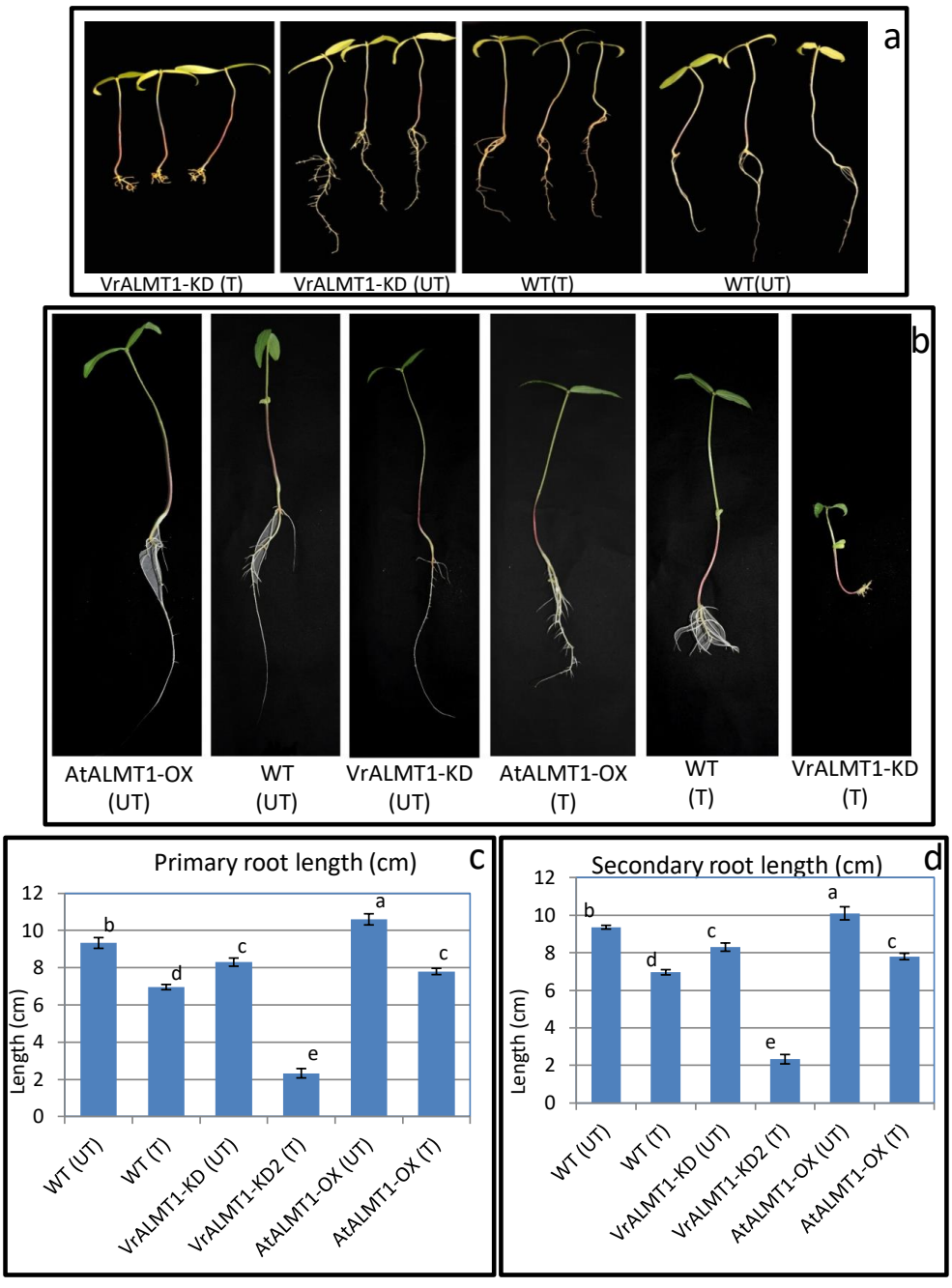

Fig.7 a-d) Variations in the growth and development of WT and transgenic seedlings under acid soil stress conditions. WT(NT), Wild type (untreated); WT(T), Wild type (Treated); VrALMT1-KD (UT), VrALMT1-Knockdown (Untreated); VrALMT1-KD (T), VrALMT1-Knockdown (Treated); VrALMT1-OX (UT), VrALMT1-overexpressors (Untreated) and VrALMT1-OX (T), VrALMT1-overexpressors (Treated). Each value indicates the mean ± SE. Mean values within a treatment followed by the same alphabets are not significant at P = 0.05 according to DMRT.

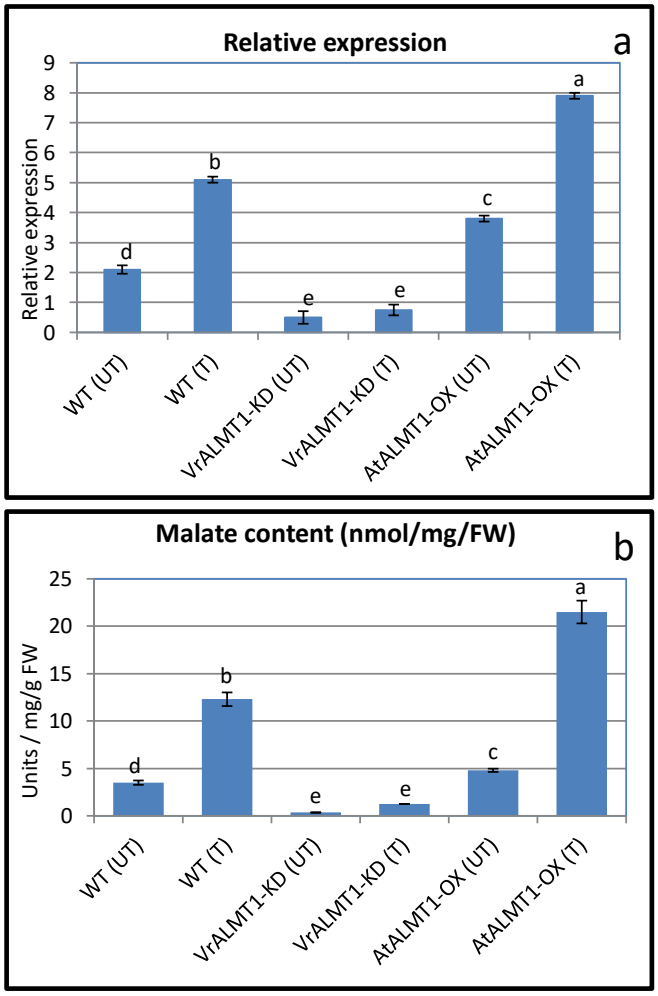

Fig.8

a) Variations in the expression profile of ALMT1 gene and b) malate content of WT and transgenic seedlings under acid soil stress conditions. WT(NT), Wild type (untreated); WT(T), Wild type (Treated); VrALMT1-KD (UT), VrALMT1-Knockdown (Untreated);); VrALMT1-KD (T), VrALMT1-Knockdown (Treated); VrALMT1-OX (UT), VrALMT1-overexpressors (Untreated) and VrALMT1-OX (T), VrALMT1-overexpressors (Treated). Each value indicates the mean ± SE. Mean values within a treatment followed by the same alphabets are not significant at P = 0.05 according to DMRT.